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Receptor-Targeted Recombinant Adenovirus Conglomerates: a Novel Molecular Conjugate Vector with Improved Expression Characteristics

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To develop improved strategies for gene transfer to hematopoietic cells, we have explored targeted gene transfer using molecular conjugate vectors (MCVs). MCVs are constructed by condensing plasmid DNA containing the gene of interest with polylysine (PL), PL linked to a replication-incompetent adenovirus (endosomolytic agent), and PL linked to streptavidin for targeting with biotinylated ligands. In this report, we compare gene transfer to K562 cells by using the previously described transferrin-targeted MCV (Trans-MCV) to a novel transferrin-targeted MCV. In the novel MCV, the transferred gene (luciferase) is in the genome of recombinant replication-incompetent adenovirus (recMCV), which also acts as the endosomolytic agent. The level of luciferase gene expression was fivefold higher in K562 cells transfected with Trans-recMCV than in cells transfected with Trans-MCV. Furthermore, targeted transfection with recMCV resulted in prolonged luciferase expression that declined 14 to 20 days after transfection, in comparison with Trans-MCV, where luciferase expression declined by 4 to 8 days. Moreover, targeted transfection of K562 cells with the Trans-recMCV resulted in persistent luciferase gene expression for 6 months. Analysis of luciferase gene expression in K562 single-cell clones that were subcloned 5 weeks after transfection with Trans-recMCV showed that 35 to 50% of the single-cell clones had intermediate to high levels of luciferase gene expression that was stable for 6 months, with the remaining clones showing low or no luciferase gene expression. Stable gene expression was associated with integration of adenovirus sequences into genomic DNA.

The goal of gene therapy is to introduce accurately and efficiently therapeutic genetic alterations into target cells. Unfortunately, current vectors and gene delivery systems are far from perfect, and significant effort is under way to improve these systems (21). One possible approach to accurately target specific cells is receptor-mediated or cell epitope-directed gene transfer. Such a targeted approach of gene transfer was first described by Wu and Wu, who used a molecular conjugate vector (MCV) directed to the asialoorosomucoid receptor of hepatocytes (36). This vector system was demonstrated by the same investigators to be functional in vivo, by targeting specifically hepatocytes in rats (35). Expression of receptor-targeted conjugate vectors can be augmented by a factor of 2,000 through the introduction of replication-incompetent adenovirus (AD) as an endosomolytic component into the MCV, where a transfection frequency of >95% was observed (32). Transferrin was used as the targetable receptor in these experiments, and other receptors or cell epitopes such as the c-*kit* receptor (26) or the immunoglobulin receptor (8) were found to be targetable with such an MCV in vitro or in vivo. Although the episomal location of the vector complex permits only transient expression with this vector system, this vector has several potential clinical applications; the transient expression of such a receptor-targeted MCV theoretically may be sufficient for targeting malignant cells with vectors encoding cytotoxic toxins or for use in immunotherapy approaches using modified TIL cells expressing cytokines or in vaccination protocols (31).

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The originally described transferrin-target MCV construct Trans-MCV (32) is currently being investigated as a vector for the interleukin-2 gene in a phase I clinical cancer immunotherapy trial for metastatic melanoma (31a). In that respect, any increased or prolonged expression property of this vector could have a significant impact on its clinical utility. The rationale for incorporating AD into the MCV so far has been solely to achieve endosomolysis and thereby protect the circular plasmid encoding a reporter gene from degradation. Although the components of the MCV provide some protection, degradation eventually will occur (4). In this context, we hypothesized that DNA encapsulated in a recombinant AD (recAD), irrespective of the infectivity of the virus, should have increased protection in the environment of an MCV. Therefore, we expected DNA degradation in the cellular environment to be delayed compared with degradation of circular plasmid DNA, thus resulting in improved expression characteristics of a reporter gene. In this work, we tested this hypothesis by constructing the transferrin-targeted recAD conglomerate vector Trans-recMCV (Fig. 1) and compared it directly to the previously described vector, Trans-MCV in the cell line used for its initial description. Trans-recMCV is constructed similarly to Trans-MCV (Fig. 1); however, recAD is linked to polylysine (PL) and condensed with salmon sperm DNA instead of the reporter plasmid. Targeting to the transferrin receptor is accomplished by using a streptavidin (SA)-PL ligand bridge in the same manner in both systems (Fig. 1). K562 cells transfected with Trans-recMCV had prolonged (6 months) and increased (fivefold) reporter gene expression compared with Trans-MCV and exhibited persistence of the reporter gene by PCR analysis. Furthermore, integration of AD sequences with

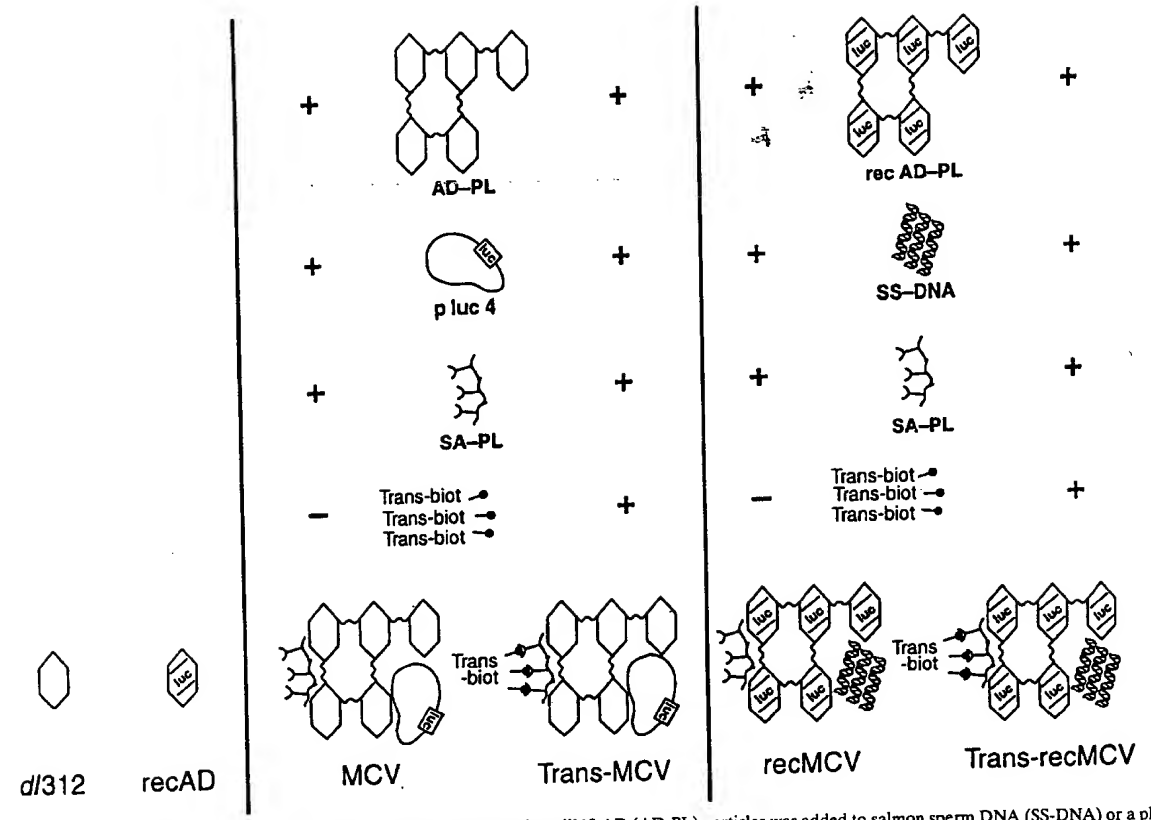

FIG. 1. Summary of MCV construction. PL linked to recAD (recAD-PL) or *dl*312 AD (AD-PL) particles was added to salmon sperm DNA (SS-DNA) or a plasmid containing luciferase (pluc4), respectively. SA-PL was added to each vector. Finally, biotinylated transferrin (Trans-biot) was added to target each vector.

the novel vector construct into the genome was demonstrated. We have compared expression characteristics of recAD to those of an equivalent amount of recAD chemically linked to PL (recMCV) and found a 50-fold increase in expression. This was not explained simply by increased uptake of the complex, which increased only fourfold. Although persistent low-grade expression in K562 cells infected at a multiplicity of infection (MOI) of >2,500 was observed, the frequency of single-cell clones expressing luciferase was very low (<1:100). Therefore, a recAD-PL conglomerate has improved expression characteristics compared with single recAD particles.

MATERIALS AND METHODS

Chemical linkage of AD and recAD to PL. The procedure was performed as previously described (26). Briefly, E1-deleted human type 5 AD (*dl*312) and E1-deleted human type 5 recAD containing the luciferase gene under a cytomegalovirus promoter (recombination of pJM17 and pAC-CMV-luc) were used to infect 293 cells (gift from Thomas Shenk, Princeton, N.J., and David Curiel, Birmingham, Ala.). Cells were harvested when a cytopathic effect was observed, and after four freeze-thaw cycles, the cell lysate was purified twice over a cesium chloride gradient by ultracentrifugation. AD was covalently linked to PL (molecular weight, 30,000 to 70,000); catalog no. 2636; Sigma, St. Louis, Mo.) with EDC [1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride; Pierce, Rockford, Ill.] to achieve a final concentration of 5×10^{11} particles/ml (1 unit of optical density [OD] at 260 nm equals 10^{12} viral particles; this equals 10^{10} PFU of prior linkage). Aliquots of AD, recAD, AD-PL, and recAD-PL were stored in viral preservation medium at −70°C (0.01 M Tris [pH 8], 0.1 M NaCl, 0.1% bovine serum albumin, 10% glycerol).

MOI. MOIs for AD *dl*312 and recAD were standardized on the basis of PFU. PFU was determined by performing plaque-forming assays using 293 cells as outlined in reference 12).

Chemical linkage of SA to PL. SA (Sigma) was linked to PL (molecular weight, 30,000 to 70,000; Sigma) as previously described (32). The SA and PL contents were determined by OD at 280 nm and OD 223 nm, respectively. PL was modified with SA at a molar ratio of 1:6.25; final concentrations were 300 μg of PL and 40 μg of SA per ml.

Plasmid DNA. The plasmid pluc4 was derived by cloning the luciferase under control of a cytomegalovirus promoter into plasmid pstcxssc, originally described by Severne et al. (28). Endotoxins were removed as previously described (19).

Cell culture and flow cytometric (FACS) analysis. K562 cells were maintained in RPMI (Gibco-Life Technologies) supplemented with 10% fetal calf serum (Atlanta Biologicals), penicillin-streptomycin (Gibco-Life Technologies), and 2 mmol of L-glutamine (Gibco-Life Technologies). Cells were passaged two to three times per week at a 1:4 ratio. Single-cell cloning was accomplished by limiting dilution into Terasaki plates (Nunc, Roskilde, Denmark) and confirmed by light microscopy. Expression of cell transferrin receptors was determined by fluorescence-activated cell sorting (FACS) analysis. Briefly, 10^5 cells were incubated with 1 μg of biotinylated transferrin (Sigma) for 60 min at 4°C, washed, and incubated with SA-fluorescein isothiocyanate (R&D Systems, Minneapolis, Minn.) for 30 min at 4°C. Cells were analyzed immediately on a Coulter (Hialeah, Fla.) Profile machine.

Preparation of vector complexes for electron microscopy. Vectors were assembled by using recAD-PL as described above except for the addition of biotinylated transferrin. In the final step of vector construction, 10 nm of streptavidinylated colloidal gold particles (Nanoprobes, Stony Brook, N.Y.) was added to the vector by using a biotin bridge; 7 μl of a 12-mg/ml stock solution of *N*-hydroxysuccinimidobiotin in dimethyl sulfoxide (Pierce) was incubated in 1 ml of 0.1 M $NaHCO_3$–0.1 M glycine solution (pH 8.5) for 1 h. One hundred microliters of this solution was added to the vector and was dialyzed against two exchanges of 1 liter of HEPES-buffered saline overnight, using a 3,000-molecular-weight-cutoff dialysis tubing (Bio-Rad, Hercules, Calif.). Control samples were processed identically without the addition of the biotin and thus lacked the biotin bridge. Twenty microliters of streptavidinylated gold (Nanoprobes) was added, and the solution was placed onto a 300-mesh Formvar film (Ladd Research Industries Inc., Burlington, Vt.)-coated grid (Ted Pella Inc., Redding, Calif.). Excess unbound gold was removed by three washes with distilled water prior to fixation with 5 μl of phosphotungstate acid (Fisher Scientific Co., Fair Lawn, N.J.). The grid was examined and photographed with an Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan). The actual sizes of the recMCV and Trans-recMCV were determined to be less than 200 nm, based on the

fact that these constructs were readily filtered through a 200-nm-cutoff filter (Millipore, Bedford, Mass.) without loss of transfection efficiency.

Transfection studies. Briefly, 100 μl of AD-PL was condensed with 2 μg of reporter plasmid, or 100 μl recAD-PL was condensed with 2 μg of sonicated salmon sperm DNA (Stratagene), for 30 min at room temperature. One lot of DNA was used with a base pair range of 300 to 2,000. The size of the preparation was confirmed by electrophoresis through 1.2% agarose. This was followed by the addition 6 μl of SA-PL and a 30-min incubation at room temperature, and these constructs served as the control vector (MCV or recMCV). The targeted vectors were constructed by adding 400 ng of biotinylated transferrin as a final step to generate Trans-MCV or Trans-recMCV (Fig. 1). Suspensions of K562 cells (10^6 cells/ml) in serum-reduced medium (2% fetal calf serum) were incubated with the MCV, Trans-MCV, recMCV, or Trans-recMCV vector or with purified recAD at different MOIs in a 1.5-ml Eppendorf tube at 37°C with rotation for 2 h. Cells were returned to their regular growth conditions in a 25-ml flask. Cells were harvested at different time points and analyzed for luciferase expression (Promega, Madison, Wis.), using a luminometer (Berthold, Bad Wildung, Germany, or Analytical Chemiluminescence, San Diego, Calif.) with a measuring time of 20 s. Relative light units (RLUs) obtained were corrected for the protein concentration of the cell lysate (Pierce) and are reported as RLU/milligram of protein.

[^{35}S]methionine labeling of recAD and recAD-PL. 293 cells were infected with recAD (10 PFU per cell), and virus was propagated in methionine-free RPMI medium (Gibco) containing [^{35}S]methionine (Amersham Life Sciences, Arlington Heights, Ill.) as previously described (27). [^{35}S]methionine-labeled recAD (^{35}S-recAD) was purified and cross-linked to PL as described above. K562 cells were infected with equal amounts (counts per minute) of ^{35}S-recAD or transfected with recMCV constructed from ^{35}S-recAD-PL. After a 2-h incubation, the cells were washed thrice with phosphate-buffered saline and trypsinized for 15 min at 37°C (Gibco) to remove any ^{35}S-recAD or ^{35}S-recMCV bound to the cell surface. The cells were washed and then pelleted. The cell pellets were dissolved in 100 μl of 0.1 M NaOH, and the radioactivity was determined by liquid scintillation counting.

PCR. DNA was extracted from cells as previously described (2). One hundred nanograms of DNA was amplified by PCR with primers specific for the luciferase gene to amplify the region between bp 772 and 1133 (sense, 5′CAA CCG CTT CCC CGA CTT C3′; antisense, 5′GTG TGG CCC TTC CGC ATA GA3′. One hundred nanograms of *Xho*I (Gibco)-digested plasmid pluc4 was used as the positive control. The amplification was performed as instructed by the manufacturer (Perkin-Elmer Cetus, Branchburg, N.J.). Amplification conditions were one cycle of 94°C for 2 min, followed by 32 cycles of 94°C for 1 min, 59°C for 30 s, and 72°C for 30 s. The PCR products were loaded and separated on a 1% agarose gel and then visualized with ethidium bromide.

Biological assay for persistence of AD vector. Supernatants and cell lysates of cell clones expressing the luciferase reporter gene were added to 293 cells grown in six-well plates (Nunc) at 70% confluency. PFU was determined as described in reference (12).

FISH. Fluorescent in situ hybridization analysis (FISH) was used to confirm the integration of AD sequences. The human AML cell line K562 was arrested in metaphase by the use of 50 ng of colcemid (GIBCO/BRL, Grand Island, N.Y.) per ml for 1 h. The cells were then exposed to 0.075 M KCl for 20 min at 37°C. The cells were fixed three times in 3:1 methanol-acetic acid at −20°C for 30 min each time and dropped onto microscope slides. The 31-kb 3′ fragment from AdCMVLacZ was obtained after *Xba*I digestion and gel purification. Construction of the virus AdCMVLacZ has been described previously (16). This fragment was labeled with biotin-16-dUTP (Boehringer Mannheim, Indianapolis, Ind.), using a large-fragment DNA labeling kit (Oncor, Inc., Gaithersburg, Md.) as instructed by the manufacturer and a 30-min incubation time. Hybridization of labeled AdCMVLacZ DNA was done for 16 h in a humidified, 37°C incubator. Detection of labeled DNA was performed by using kits from Oncor. The signals were observed on a Zeiss Axioskop epifluorescence microscope, using a 63× Apo-Chromat (numerical aperture = 1.4) or 100× Apo-Chromat (numerical aperture = 1.4) (Carl Zeiss Inc., Thornwood, N.Y.) iris. The images were captured with a ZVS 47DEC CCD camera (Carl Zeiss) and Image Central software (Advanced Imaging Concepts, Princeton, (N.J.).

RESULTS

Binding of biotinylated ligand to recMCV. To demonstrate the presence and amount of the biotinylated ligand contained in recMCV, biotinylated transferrin was substituted with streptavidinylated colloidal gold particles by using a biotin bridge in the final step of vector construction. Examination of these vector complexes by electron microscopy showed the expected AD morphology (Fig. 2A) in the molecular vector complex as well as the bound colloidal gold particles (Fig. 2B). Significant accumulation of the colloidal gold is seen coating the recombinant conglomerates, while the control conglomerates lacking the biotin bridge do not contain colloidal gold particles (compare Fig. 2A and B). Thus, there is effective binding of biotinylated ligand to the SA sites in the recMCV complex, and recAD can be chemically linked to PL.

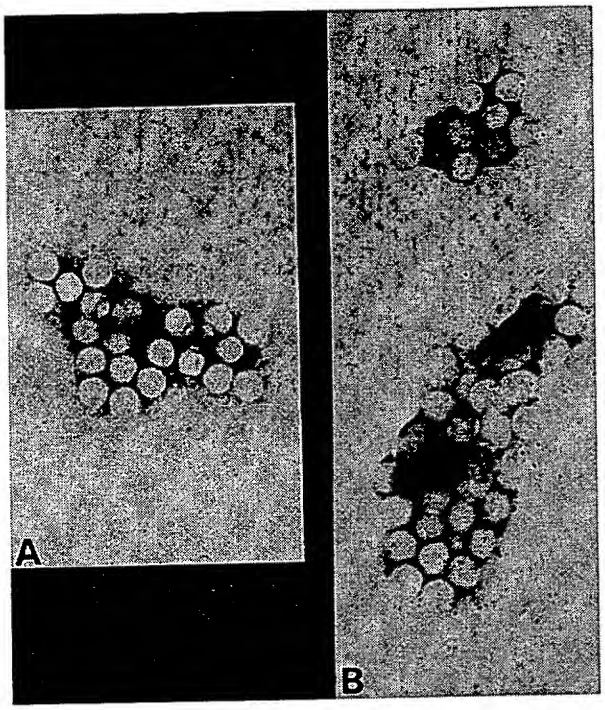

FIG. 2. Electron microscopy of recMCV. recMCV was constructed as described in Materials and Methods. Streptavidinylated gold particles were added to recMCV in the final step of construction as described in Materials and Methods (B). The control MCV is seen in panel A.

Transferrin receptor expression on K562 cells. High expression of the transferrin receptor on K562 cells was confirmed by FACS analysis (data not shown).

Comparison of luciferase gene expression in K562 cells transduced with recAD and the MCV constructs. "Transferrinfection" is a transferrin receptor-mediated gene transfer method originally described by Wagner et al. (32) that condenses plasmid DNA encoding the luciferase reporter gene with AD-PL as part of the vector construction (26). To improve the efficiency of targeted gene transfer, we compared a vector prepared by transferrinfection to a novel transferrin-targeted vector that was assembled with recAD containing the luciferase gene (Fig. 1). Briefly, equivalent amounts of E1-deleted *dl*312 AD or luciferase gene-encoding recAD were linked to PL to generate AD-PL and recAD-PL, respectively. Then, plasmid DNA was added to AD-PL and salmon sperm DNA was added to recAD-PL. This was followed by the addition of SA-PL to both constructs to generate MCV and recMCV, respectively. These vectors are also referred to as the untargeted or control vectors. The addition of biotinylated transferrin as a final step to either system-generated transferrin receptor-targeted MCVs (Trans-MCV and Trans-recMCV). Therefore, the vectors contain either AD-PL (MCV or Trans-MCV) or recAD-PL (recMCV or Trans-recMCV). Finally, both vectors contained the equivalent of 500 PFU of AD or recAD per cell prior to linkage with PL. K562 cells were transfected with the transferrin-targeted vectors Trans-MCV and Trans-recMCV, which resulted in luciferase gene expression fourfold above the level for control vectors at 48 h (MCV and

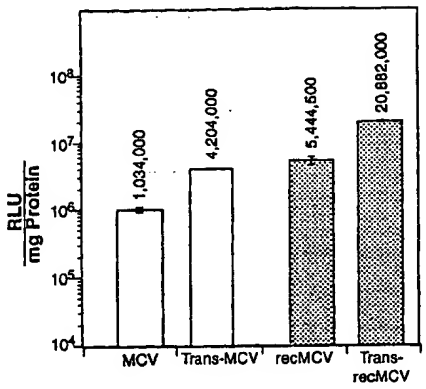

FIG. 3. Effect of recAD on transferrin-targeted transfection of K562 cells. K562 cells were transfected with the control or the transferrin-targeted MCV or to the control or the transferrin-targeted recMCV, as indicated and as described in Materials and Methods, and analyzed at 48 h. The data are presented as mean RLU of duplicate determinations ± standard error and are representative of three experiments.

recMCV, respectively) (Fig. 3; compare bars 1 and 2 and bars 3 and 4). Interestingly, there was a fivefold increase in luciferase gene expression in both the recMCV and the Trans-recMCV vector over the level for the MCV counterpart (Fig. 3; compare bars 1 and 3 and bars 2 and 4). Thus, expression of the transferred gene is significantly greater with the novel vector Trans-recMCV than in the previously described transferrinfection system.

Next, we wanted to compare reporter gene expression in K562 cells that were infected with recAD particles to cells transduced with an equal amount of PFU of recAD chemically linked to PL. Therefore, K562 cells were infected at an MOI of 500 PFU with recAD particles or recAD complexed in the molecular conjugate (MOI of 500 PFU). Reporter gene expression was seen to be 50-fold higher after recMCV infection than after recAD infection at 48 h (Fig. 4A). Thus, luciferase gene expression is significantly higher when recAD is complexed in the MCV than with an equivalent amount of recAD particles.

Comparison of uptake of recAD particles and recMCV by K562 cells. Since increased viral particle uptake in the MCV form could explain improved gene expression over recAD particles, recAD was labeled with [^{35}S]methionine and then linked to PL. K562 cells were incubated with equivalent amounts (counts per minute) of recAD complexed in the MCV or as free particles for 2 h, washed, trypsinized, and analyzed for radioactivity (Fig. 4B). While 4.7% of the total counts of ^{35}S-recAD was found associated with the cells infected with recAD particles, there was an approximately fourfold (17%) increase in uptake in assays using equivalent amounts of ^{35}S-recAD complexed in the MCV. Thus, while there is a 50-fold increase in luciferase gene expression when recAD is contained in the MCV compared to the recAD particles, there is only a 4-fold increase in uptake of recAD particles associated in K562 cells.

Comparison of the kinetics of luciferase gene expression in K562 cells transfected with recAD and with transferrin-targeted Trans-MCV versus Trans-recMCV. We determined luciferase gene expression and the kinetics of luciferase gene expression as a function of MOI by infecting K562 cells at increasing MOIs of recAD. K562 cells were infected with recAD at MOIs of 80, 160, 320, 640, 1,280, 2,560, and 5,120 PFU. Maximum expression was observed after 36 h in all experiments. Expression returned to baseline at 5 weeks in cells infected with MOIs of up to 1,280 PFU. Increased reporter gene expression was seen with increasing MOIs up to 2,560 PFU but could not be further increased by doubling the MOI to 5,120 PFU (Fig. 5A). Interestingly, low levels of luciferase gene expression were observed over a period of 6 months in cells infected with the highest MOIs (2,560 and 5,120 PFU).

We next compared the kinetics of luciferase gene expression in K562 cells transfected with Trans-MCV or Trans-recMCV. As previously demonstrated, targeted transferrin transfection (Trans-MCV) results in transient gene expression in K562 cells that declines by 4 to 8 days in culture and returns to baseline expression thereafter (Fig. 5B). We have occasionally observed persistent expression at very low levels over prolonged periods (data not shown). In contrast, cells transfected with Trans-recMCV showed prolonged expression of the transferred gene that did not decline until 14 to 20 days in culture. Furthermore, reduced but persistent reporter gene expression was observed over the 6-month period examined, suggesting stable expression. The kinetics of luciferase gene expression were identical for the untargeted and control conjugate vectors, MCV and recMCV, respectively, although at proportionally decreased levels as demonstrated in Fig. 3 (data not shown). Thus, increasing the MOI of free recAD particles results in increased reporter gene expression that reaches a maximum at an MOI of 2,500. Although the kinetics of luciferase gene expression at these very high MOIs of recAD are similar to the kinetics seen with Trans-recMCV, gene expression achieved with Trans-recMCV is greatly enhanced. Gene expression is prolonged and is stable (albeit at lower levels) for 6 months.

Establishment and characterization of stably transfected K562 cells with persistent luciferase gene expression. Since persistent expression was observed in cells transfected with Trans-recMCV, we wanted to determine the frequency of stable transfection to individual cells. Therefore, K562 cells transfected with Trans-recMCV were cloned by limiting dilution analysis 5 weeks after the initial transfection. Luciferase gene expression was determined in 100 single-cell clones in two experiments and was categorized as high, intermediate, or low (see the footnote to Table 1). The results of two experiments each examining 100 single-cell clones obtained from K562 cells transfected with Trans-recMCV after 5 weeks in culture (Table 1) show that 35 to 50% of the single-cell clones continue to

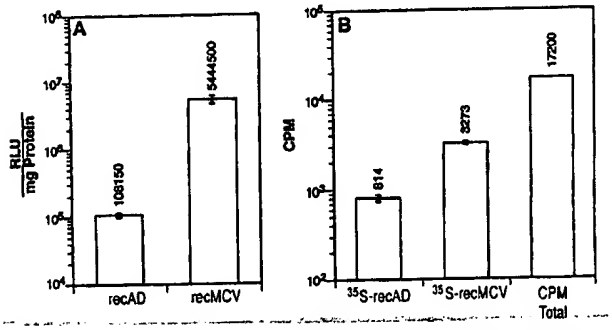

FIG. 4. (A) Comparison of luciferase gene expression in K562 cells infected with an equivalent amount of recAD (500 PFU) or transfected with recAD-PL (500 PFU of prior chemical linkage) contained in the MCV. The data are presented as mean RLU, measured at 48 h, of triplicate determinations ± standard error and are representative of two experiments. (B) Comparison of the uptake of ^{35}S- recAD particles and ^{35}S- recAD conjugated in the MCV on K562 cells. K562 cells were incubated with equivalent amounts of radioactivity as ^{35}S-recAD or ^{35}S-recAD-PL contained in the MCV as described in Materials and Methods. CPM Total, total amount of radioactively labeled recAD added to K562 cells.

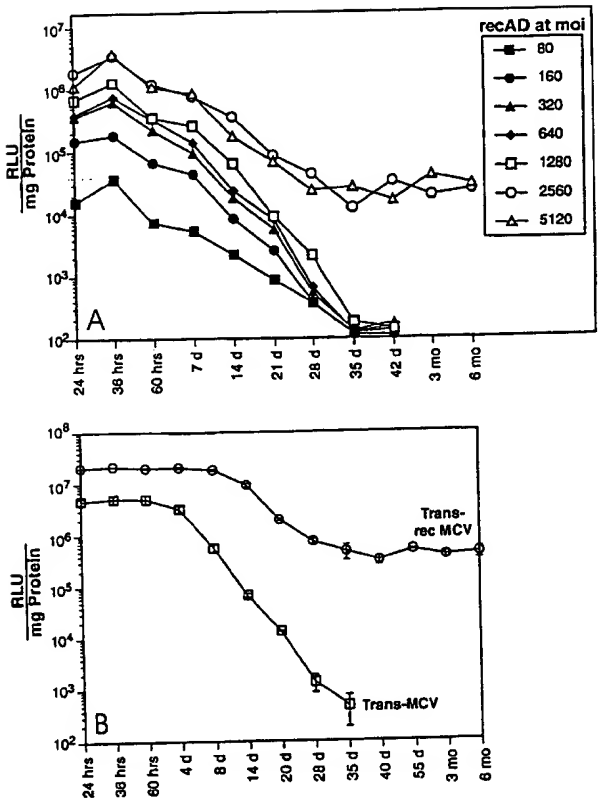

FIG. 5. Kinetics of luciferase gene expression of recAD at increasing MOIs, of Trans-MCV, and of Trans-recMCV. K562 cells were infected with increasing MOIs of recAD and analyzed sequentially over a 6-month period for luciferase gene expression (A). K562 cells were transfected with Trans-MCV or Trans-recMCV in triplicate as described in Materials and Methods. Luciferase gene expression was determined sequentially over a 6-month period as outlined in Materials and Methods (B). The data are presented as mean RLU and are representative of two separate experiments.

have intermediate to high levels of luciferase gene expression. In contrast, we were unable to obtain single-cell luciferase gene-expressing clones from K562 cells infected with recAD particles in two separate experiments each examining 100 single-cell clones. Therefore, we determined the efficiency of recAD stably transduced K562 cells to be less than 1%. Thus, transfection of K562 cells with the novel vector Trans-recMCV results in variable expression levels ranging from zero to greater than 10-fold for individual single-cell clones compared

TABLE 1. Reporter gene expression[a]

Expt	Reporter gene expression (RLU/mg of protein)			
	High	Intermediate	Low	None
1	5	45	10	40
2	6	29	17	48

[a] Reporter gene expression was measured in single-cell clones obtained from K562 cells at the time of steady-state expression. Parental K562 cells were transfected with Trans-recMCV and analyzed for receptor gene expression. Expression category was assigned arbitrarily as high (expression >10-fold that of the parental clone), low (expression <1-fold that of the parental clone), or intermediate (expression 1- to 10-fold that of the parental clone). Nonexpressing clones were zero by definition. The results of two separate experiments, each analyzing 100 clones, are shown.

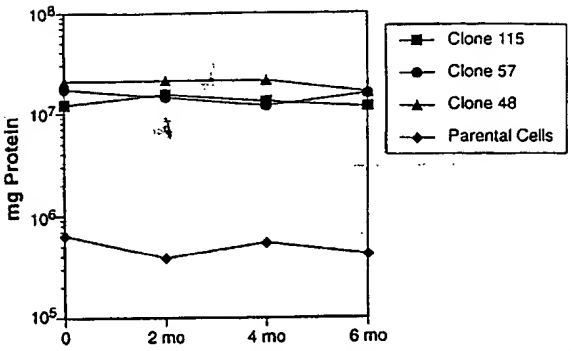

FIG. 6. Stable luciferase gene expression in high-expressing single-cell K562 clones. K562 cells were transfected as outlined in Materials and Methods with Trans-recMCV and then subcloned by limiting dilution analysis 5 weeks after the initial transfection. Luciferase gene expression was determined over a 6-month period on three high-expressing clones and the parental transduced clone. The data are presented as RLU of individual cell clones and are representative of two separate experiments.

to the parental cells. Taken together, the data indicate that Trans-recMCV is a high-efficiency novel gene transfer vector resulting in stable luciferase gene expression over a 6-month period that occurs with relatively high frequency (35 to 50%) in K562 cells subcloned 5 weeks after initial transfection in culture.

Next, we determined the expression of luciferase in high-expressing single-cell clones over time. Three high-expressing single-cell clones obtained from the experiment described above were analyzed for reporter gene expression every 2 months over a 6-month period (Fig. 6). The expression of luciferase was stable over the 6-month period for all of the high-expressing clones examined. The expression of luciferase from the transfected parental cells is shown for comparison. Furthermore, expression of luciferase in the intermediate- and low-expressing clones was also stable over a 6-month period (data not shown).

Presence of the luciferase gene, absence of episomally retained recAD, and genomic integration of AD sequences in luciferase gene-expressing cell lines transduced with Trans-recMCV. To demonstrate the presence of the luciferase gene in Trans-recMCV-transfected K562 cells, we examined three high-expressing luciferase clones (clones 115, 57, and 48) and three clones that did not express luciferase (clones 61, 46, and 33) by PCR using specific primers for the luciferase gene. A specific 361-bp segment was amplified in luciferase-expressing clones (Fig. 7, lanes 2 to 4) but not in clones that did not express luciferase (lanes 5 to 7). The amplified band was the same as that seen for the luciferase gene contained in plasmid pluc4 (lane 1). Propagation in transduced cells of free, episomally retained recAD may result in both prolonged reporter gene expression and a positive PCR signal for the luciferase gene. Therefore, we examined the supernatants and cell lysates of 12 different cell clones that were expressing the luciferase reporter gene at high levels (as defined above) for the presence of AD, using a biological assay at 3 and 6 months posttransfection. Sensitivity of the assay was determined by observing a cytopathic effect with vector at a level as low as 10 particles/ml of medium. None of these assays resulted in plaque formation and thus were negative for the presence of AD.

Finally, we wanted to determine if AD sequences are present in the genomes of the high-expressing transduced cell clones 48, 57, and 115. FISH analysis was performed with the

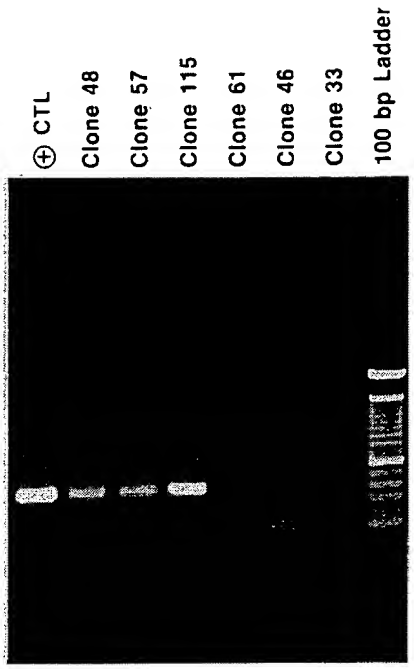

FIG. 7. Detection of luciferase DNA by PCR analysis in single-cell clones obtained from K562 cells transfected with Trans-MCV. Three high-luciferase-expressing clones (clones 115, 57, and 48) and three nonexpressing K562 clones were obtained as described in Materials and Methods. DNA obtained from these clones was amplified by PCR for a 361-bp fragment specific for the luciferase gene as described in Materials and Methods. Linearized plasmid pluc4 DNA containing the luciferase gene was used as the positive control (CTL).

31-kb 3′ AD fragment, and hybridization of the probe to chromosomes of each transduced cell line was demonstrated (Fig. 8 and data not shown). Thus, AD sequences integrated into only one chromosomal homolog in each cell. This is most obvious by the single signal detected in interphase nuclei (Fig. 8D). As a control, uninfected K562 cells were treated in parallel. No signal was detected in uninfected K562 cells (data not shown). Thus, the stable expression accomplished with the novel vector Trans-recMCV in K 562 cells is associated both with persistence of the luciferase gene and integration of AD sequences into the genome of the transduced cells.

DISCUSSION

In this work we have compared a modified gene transfer system with a previously described transferrin receptor-targeted MCV in the human erythroleukemia cell line K562 (39, 40). Delivery of DNA in this novel vector is accomplished by targeting recombinant AD-PL conglomerates to the transferrin receptor via vector-bound transferrin ligands. Effective binding of transferrin to recMCV was visualized with electron microscopy, although the conditions chosen for this procedure were different from the conditions for the actual transfection experiments. Therefore, these experiments may serve only as an estimate for the final size of the complex. Effective binding of the biotinylated ligand to recMCV was also validated in competition assays with excess ligand and specific monoclonal antibodies as previously described by others and us (references 20 and 26 and data not shown). Although comparison of recAD and the same quantity complexed as recMCV in conglomerate form seems acceptable, the Trans-MCV system used E1-deleted *dl*312 AD solely for the purpose of endosomolysis. Moreover, in the Trans-MCV system, the reporter gene is in *trans*, bound to the vector complex as a circular plasmid by electrostatic forces. In comparison, the novel vector presented here combines the function of DNA delivery with endosomolysis in *cis*. The PFU-to-viral particle ratio after virus purification was 1:100. A significant inactivation with the chemical linkage procedure is reported, and therefore the number of infectious virus in Trans-recMCV or Trans-MCV is assumed to be far lower after linkage (2×10^5 viral particles per PFU) (9). In our experiments, AD was controlled for particle number since it has been established that chemical modification reduces infectivity as measured by plaque formation. However, it has not been demonstrated that chemical modification affects endosomolytic properties (5). DNA transfected with Trans-MCV is believed to remain episomal, and therefore intracellular degradation and mitosis will result in transient expression. We have demonstrated that Trans-recMCV expression levels are significantly higher than those of Trans-MCV at any time point (Fig. 5B). A rapid decline in the expression of episomal contained reporter genes to baseline after 2 weeks would be expected in cells maintained in culture due to dilution by dividing cells. This effect is seen in cells transfected with Trans-MCV. Although significantly delayed, expression will decline in Trans-recMCV-transfected cells and reach a constant baseline expression at 5 weeks, whereas no expression is observed in Trans-MCV-transfected cells at that time point.

This improved early expression (<30 days) of Trans-recMCV compared with Trans-MCV could be explained by the delivery of DNA as recAD conglomerates to confer increased stability over DNA delivered in plasmid form. However, this would not explain the persistence of the reporter gene by PCR analysis and the persistence of expression at constant levels over a 6-month period unless integration of the reporter gene into the host genome occurred. In contrast, the reporter gene was not detectable after cessation of expression in Trans-MCV-transfected cells by PCR analysis, indicating complete disappearance of this sequence (data not shown). Using FISH analysis, we have demonstrated integration of AD sequences into the genome of high-expressing cell clones that are transduced with the Trans-recMCV vector. Hybridization of the probe was seen in single homologs of the different clones, indicating integration events of AD sequences to be only single events. Persistence and propagation of E1-deleted recAD in human bone marrow cells and leukemia cell lines has been reported previously and is an alternative explanation for persistent gene expression (22). We were unable to detect free AD in high-expressing clones. Furthermore, incompletely deleted and propagating recAD would not be expected to result in integration into genomic DNA. Additional reasons against the propagation of recAD as a cause for the persistent expression are the threshold effect seen with recAD (Fig. 5A) and the presumed decreased infectivity of recAD in conglomerate form caused by the chemical linkage procedure as discussed above.

Our data suggest that the ability to integrate is a function of the reporter DNA presented as a recAD conglomerate since no integration events were seen with the Trans-MCV system for DNA delivered as plasmid. To demonstrate this correlation further, we exchanged in our transfection studies salmon sperm DNA in the Trans-recMCV for a *lacZ*-encoding plasmid. Although short-term expression of the *lacZ* gene was observed, the gene could not be detected 2 weeks after cessation of gene expression by PCR analysis. However, expression of the AD-encoded luciferase gene was stable and followed expression kinetics reported for Trans-recMCV in Results. Although there may be concern for potential clinical use of

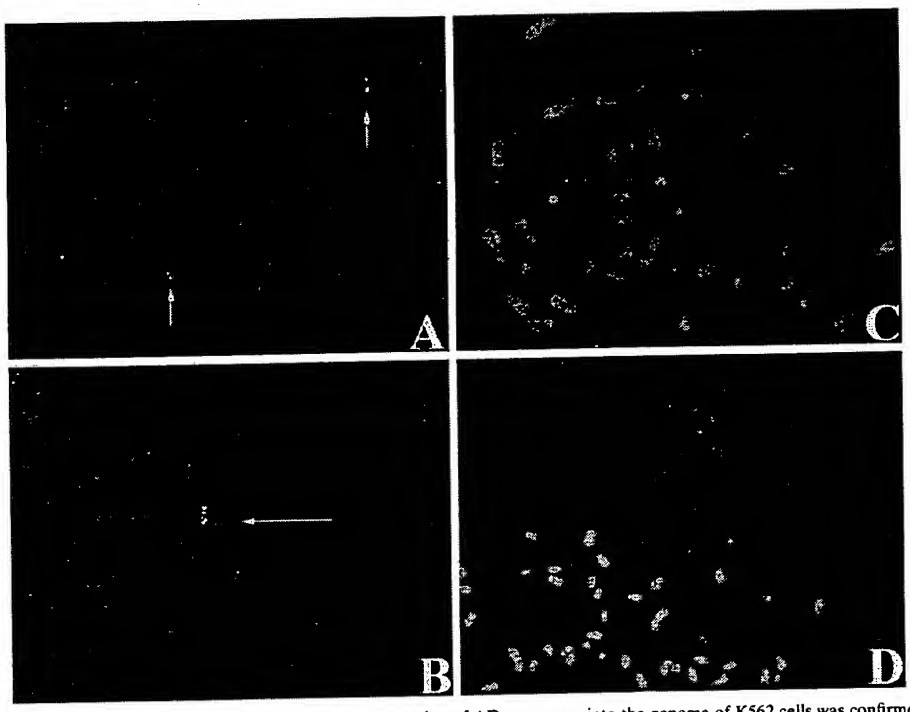

FIG. 8. FISH analysis of high-expressing single-cell clones. The incorporation of AD sequences into the genome of K562 cells was confirmed by FISH. The 31-kb 3′ end of AdCMVLacZ DNA was labeled with biotin as described in Materials and Methods and detected with either fluorescein isothiocyanate-avidin (A and B) or Texas Red-avidin (C and D). (A and B) The signals detected in clone 115 with propidium iodide counterstaining (arrows); (C and D) the signals detected in clone 48 with 4′,6-diamidino-2-phenylindole counterstaining (arrows). Although the entire nucleus cannot be seen in panel D, only one signal was seen. The chromosomes in panel A were derived from two cells; those in all other panels were derived from one cell. Note that in panel A, two overlapping metaphase spreads are represented. Thus, the AdCMVLacZ sequences integrated into only one chromosomal homolog in each cell. This is most obvious by the single signal detected in interphase nuclei (D). As a control, uninfected K562 cells were treated in parallel. No signal was detected in uninfected K562 cells (data not shown).

vectors containing salmon sperm DNA, this reagent served only to control for charge neutralization between the different MCVs since cationic charges are associated with increased cellular uptake of gene transfer vectors (1, 7).

It appears that the formation of recAD conglomerates by themselves confers increased stability over recAD, since the improved expression characteristics of recMCV over recAD (50-fold) cannot be explained by increased uptake alone (four-fold). Although as a caveat, a standard assay was used to measure internalization (27), trypsin may not efficiently disrupt PL-membrane interactions, and therefore the data presented may overestimate the actual recMCV internalization. The four-fold increased uptake could be caused by uptake of conglomerates via infective viral particles present, but in reduced amounts, in the conglomerate, via the PL component, or by a combination of both (1, 7, 20, 30, 34).

This speculation is supported by the observation of a dose response with increasing MOIs of recAD (Fig. 5A) until a maximum is achieved (2,560 PFU). Although significant levels of gene expression were observed with recAD at this maximum, recMCV expression levels were substantially higher (20-fold). Limited numbers of receptors on cells could explain this maximum. Stable low levels of reporter gene expression are seen at these excessive MOIs, and there seems to be a threshold to achieve this effect. Although these stable expressing recAD-infected cell populations remained PCR positive for the luciferase gene, we were unable to obtain single-cell expressing clones, and the screened clones were also PCR negative for the luciferase gene. Therefore, the transduction frequency obtainable with recAD in K562 cells appears to be very low. The low transduction efficiency might conceivably be explained solely through limited virus uptake determined by receptor number, which is reported to be relatively low in K562 cells (33). However, we believe that this low transduction efficiency may not be directly related to AD receptor expression. In this regard, in cells overexpressing AD receptor at high levels, AD infection results in very low stable integration events in the order of 10^{-6} (11, 17). Therefore, vector- or cell-related mechanisms in addition to AD receptor expression and uptake may affect reporter gene expression and integration efficiency.

The binding of transferrin to the vector for the purpose of targeting adds an additional entry route to this vector system that will compete for uptake with the PL-mediated and AD-mediated mechanisms. Thus, with this vector system, there may be significant differences among cell lines that express AD and transferrin receptors at various levels in addition to individual variability between cells regarding charge-related cell-vector interactions (1, 7, 20, 30, 34). Therefore, it is possible that in some cells, one mechanism for vector uptake may override the other mechanisms.

In this regard, Michaël et al. showed that transferrin-coated AD-PL-DNA complexes can be effectively targeted to the transferrin receptor in AD receptor-overexpressing HeLa cells, although vector entry occurred via the AD receptor as well. This background could be blocked with specific anti-AD antibodies (20). We were able to validate and reproduce these authors' data for the Trans-recMCV system in HeLa cells, and we demonstrated a transferrin receptor-mediated enhancement of reporter gene expression (data not shown). These data

suggest that the targeting properties of the novel Trans-recMCV are similar or identical to those of the Trans-MCV vector in K562 and in HeLa cells.

In addition to the endosomolytic properties of the virus capsid that protects virus DNA from digestion, Chiou et al. have demonstrated decreased degradation of plasmid DNA in a PL conglomerate (4). We speculate that DNA AD particles in a PL conglomerate may have additional increased protection from degradation over individual particles. We hypothesize that delivery of intact DNA to the nucleus is improved using the recMCV system.

From our data, we conclude the ability to integrate into the host genome of K562 cells to be a property of this novel, recombinant MCV. Further characterization of the mechanism and pattern of the integration events is required.

We also have performed experiments in the CD34-expressing hematopoietic progenitor cell lines MBO2 and M-O7e, using the novel recMCV vector. We confirmed in these cell lines gene expression significantly improved over that seen with the MCV system. Moreover, we demonstrated that one can take advantage of the c-*kit* receptor for vector entry by conjugating biotinylated c-*kit* ligand (stem cell factor) to the recMCV. The c-*kit* receptor is highly expressed on these progenitor cells (data not shown). Similar to our previously published data with the MCV, these experiments targeting different receptors demonstrate the versatility of the system (26). Although short-term gene expression was significantly enhanced with use of the recMCV vectors, gene expression kinetics at the protein level were not significantly prolonged in either cell line compared to the previously published MCV. However, we demonstrated persistence of the luciferase gene in both cell lines transfected the novel recMCV vector by PCR out to 3 months. In contrast, cells transfected with the original MCV system or recAD alone demonstrated rapid loss of the luciferase reporter gene shortly after cessation of gene expression (within 2 weeks of transfection) (data not shown). We interpret these results as a cell line-dependent transgene shut off (transcriptional or translational) despite persistence of the gene, and this phenomenon is currently under investigation in our laboratory. Preliminary experiments in CD34-selected human bone marrow cells that express both the transferrin and the c-*kit* receptor have shown infection and reporter expression on a short-term basis with both the MCV and recMCV. Like most transfection procedures, it was not as efficient in primary cells as in cell lines. However, it was significantly improved over that with recAD, which poorly infects this cell type due to lack of receptors (3). In these preliminary experiments, targeting the transferrin or the c-*kit* receptor did not result in the receptor-mediated boost in gene expression.

AD vectors have important potential application in various gene therapy approaches such as those used for cystic fibrosis (24) or other inherited disorders such as familial hyperlipidemia and hypercholesterolemia, where temporary correction of this condition was demonstrated in vivo (18, 29). However, transfection efficiency and expression with recAD vectors is tissue dependent and only transient expression is observed, since the vector does not integrate (14, 21). Immune responses to reporter genes and to AD proteins coexpressed in transfected cells have been found to be a significant problem in in vivo studies with preferential elimination of infected cells with first-generation recAD vectors (6, 37). This may explain the results in clinical trials, which did not provide a clinical benefit at the present stage of investigation (15). Crippling the AD vector and the design of helper virus-dependent vectors are current strategies to circumvent these problems and to generate a less immunogenic vector with a higher therapeutic index (23, 38). The principle of targeted recAD conglomerates should also be applicable to such improved AD vectors and may affect further development in the AD vector field. In addition, the concept of forming recAD conglomerates combined with endosomolysis can be extended to other recombinant viral vectors. For instance, retroviral vectors could be chemically linked to PL by using a similar procedure previously described by Hiroshi et al., who chemically linked the asialoglycoprotein ligand to ecotropic retroviral vector particles, thereby redirecting tropism of the virus and possibly conferring improved intracellular survival (13). Alternatively, an antibody biotin-SA linkage technique has been reported by Roux et al. (25). Such a retrovirus-PL construct could be condensed with DNA and AD-PL for additional endosomolysis and could be targeted to a receptor in a way similar to that reported in this work. Although it is possible to target recombinant retroviral vectors via a ligand epitope expressed in the envelope of the vector (10), additional problems such as very low titers and low efficiency are limiting this system at the present stage of its development (25). Since retroviral particles are very labile and unsuitable for most in vivo applications due to immediate degradation, any manipulation conferring increased stability could significantly improve potential clinical applications. It is possible that such a targeted conglomerate construct will have greater stability than single-vector particles. Although this work outlines a novel concept of gene transfer, further experiments have to be conducted with other ligands and other cell lines to explore the utility and application of this system for different conditions. The results presented provide the rationale to conduct such experiments to develop and to test this gene transfer concept consisting of recombinant vector conglomerates in conjunction with endosomolytic agents.

In summary, we have demonstrated improved expression characteristics for a novel targeted AD conglomerate vector compared with a previously described targeted MCV in the K562 cells. Although DNA degradation was not measured directly and the conclusions are based on gene expression data, it appears that reporter DNA in recAD has increased protection from intracellular degradation compared with plasmid DNA, and the formation of recombinant AD-PL conglomerates confers increased stability over single recAD particles. Moreover, this stable expression was associated with integration of AD sequences into genomic DNA. This novel concept of targeted vector complexes with or without endosomolytic agents could be explored further with various existing recombinant vectors.

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